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Bioaccumulation and Partitioning of Heavy Metals in *Cicindelidia haemorrhagica* in Yellowstone National Park

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BIOACCUMULATION AND PARTITIONING OF HEAVY METALS IN CICINDELIDIA HAEMORRHAGICA IN YELLOWSTONE NATIONAL PARK

by

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BIOACCUMULATION AND COMPARTMENTALIZATION OF HEAVY METALS IN *CICINDELIDIA HAEMORRHAGICA* IN YELLOWSTONE NATIONAL PARK

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The tiger beetle *Cicindelidia haemorrhagica* (Coleoptera: Cicindelidae) are widely distributed in Yellowstone National Park (YNP) and exclusively living on thermal areas. Heavy metals including arsenic, copper, lead, and selenium are present in these thermal springs, presenting an unique environment for *C. haemorrhagica*. Therefore, from 2018 – 2020 I sampled adult *C. haemorrhagica* from YNP and adult *C. haemorrhagica* from a known population in a salt flat in Idaho not associated with a hot spring and measured heavy metal concentrations. All populations of *C. haemorrhagica* bioaccumulated heavy metals. Cuticular waxes showed small concentrations of metals indicating that those metals are being excreted. The exoskeleton and internal tissues had much greater concentrations indicating bioaccumulation in the exoskeleton and possible bioaccumulation in the internal tissue. Metal concentrations in the exoskeleton were different between metals, but the most statistically significant differences were for arsenic and selenium. Differences in arsenic distribution occurred in YNP beetles, compared to Idaho beetles raising the question of whether *C. haemorrhagica* in YNP have so diverged in adaptations from their counterparts in Idaho in regard to heavy metals associated with thermal areas that they may represent a distinct species or subspecies.

DEDICATION

I dedicate this thesis to my parents, Dr. Matt and Michele Gotschall, for their unwavering love and support. Thank you for your love, encouragement, and advise not only throughout my masters, but in everything I do in life. I would not be who I am without you. I love you both.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

Cicindelidia haemorrhagica was first recorded in 1891 in Yellowstone National Park (YNP) (Hubbard, 1891). While visiting YNP in 2006, L. G. Higley noticed *C. haemorrhagica* at Mammoth Hot Springs (Willemssens 2019). This led to research that established that beetles were distributed relatively widely in YNP in association with thermal areas (Willemssens 2019). Although toxins (arsenic, copper, hydrogen sulfide, methyl-mercury and sulfur, as well as radioactive materials) are known to be present in these thermal areas (Inskeep & McDermott 2005), there is little known about how animals living within those thermal features tolerate potentially lethal amounts of metals. Most research in YNP dealing with heavy metals ("naturally occurring elements with a high atomic weight and at least 5 times greater than that of water") (Tchouwuo et al. 2012) in extremophiles (organisms that thrive in "extreme environments" under high pressure and temperatures such as hydrothermal vents) has focused on bacteria, algae, and Archaea that are associated with thermal pools (McDermott 1999). Research on heavy metals in megafauna, such as moose, grizzly bear, elk, and fish, has also been conducted inside YNP. However, other than brine flies (Diptera: Ephydridae), virtually nothing is known about the thermophilic invertebrates that spend their entire lives within their "extreme environment".

The chemistry and high temperatures are main factors in why the thermal features in YNP are dangerous. Organisms adapted to these areas must accommodate not only high temperatures, but also a diverse range of potentially poisonous heavy metals.

Relatively few eukaryotes are associated with the thermal areas in YNP, but from observations in 2016 – 2019, *C. haemorrhagica* is one of the more noticeable eukaryotic extremophiles and most likely the apex invertebrate predator associated with the thermal pools (Willemssens 2019). Outside of YNP, *C. haemorrhagica* is not exclusively associated with thermal pools, which caused us to question how *C. haemorrhagica* inside YNP became adapted to living with the abnormal environmental elements. Willemssens (2019) found a difference in thermal regulating behaviors between adult *C. haemorrhagica* outside and inside YNP, therefore, opening up the question as to what else is different between the two groups because of their adaptations to different environmental stressors, such as varying pH, high temperatures, and heavy metals concentrations.

Initial testing of *C. haemorrhagica* in 2017 by Willemssens and the McDermott, Peterson, and Higley labs, indicated that adults were bioaccumulating metals from their environment; "bioaccumulation occurs when the compound concentrates in living organisms or tissues" (Kodavanti et al. 2014). The tests showed whole-body levels of arsenic, lead, mercury, antimony, cadmium, chromium, copper, iron, manganese, and magnesium. These, in addition zinc and potassium, also were found in the soil and water. With these results, and nothing in the literature on YNP or *C. haemorrhagica* pertaining to our findings, I became interested in examining *C. haemorrhagica* and heavy metal accumulation in more detail. More specifically, how does *C. haemorrhagica* detoxify, excrete, or sequester heavy metals, and in which tissues of the beetle (if any) are metals accumulated. Consequently, the purpose of my work is to measure (in an Idaho and two

YNP populations of adults) heavy metal bioaccumulations and metal distribution in different tissues.

Cicindelidae

There are more than 2,700 species of tiger beetles throughout the world varying in size and color. Due to their diverse body colors, markings, and their unique behavior it comes as no surprise that they are one of the more studied insects by entomologists and entomology enthusiasts (Duran & Gough 2020). They are found on almost every land surface other than Antarctica, the Arctic, and isolated oceanic islands (Pearson et al. 2015). Of the 116 species, and 153 possible subspecies in North America, there are 14 genera of tiger beetles (Pearson et al. 2015).

Adult cicindelids can vary in body size, color, and shape and number of markings. Therefore, in the field, the easiest way to recognize them is from their run-stop behavior (Lenard & Bell 1999). When hunting for prey, or finding a mate, an adult tiger beetle's vision blurs due to running so fast that it visually cannot process the changing light images in its brain (Lenard & Bell 1999). This makes the beetle stop to refocus, change direction and/or continue after what they are chasing. However, their speed makes up for them having to stop often, resulting in outrunning prey species for easy capture. Prey items can include ants, spiders, flies, and other arthropods as big or smaller than the tiger beetles. When a tiger beetle is successful in capturing its prey with its mandibles, it chews the prey, excreting enzymes to dissolve the tissues causing the prey to become a mushy paste the tiger beetle then can slurp up (Leonard & Bell 1999).

Adults are mostly found in open areas without vegetation such as heaths, dunes, semi-arid regions, and riverbanks (Dreisig 1980). They also bury themselves in the soil

from 15 to 30 cm for protection while resting. Depending on the species, adult burrows can differ, which help indicate what tiger beetle species is present (Pearson et al. 2015).

Tiger Beetle Morphology

The cuticle, or exoskeleton, covers the entire tiger beetle and acts as armor for protection from mechanical injury and desiccation, and for biologists it aids in species identification. Because of different patterns of micro-sculptures on the outermost layer of cuticle, we are able to identify the various species and genera. Additionally, on top of the cuticle layer are translucent waxes, made of hydrocarbons and fatty acids, that reflect light (Pearson & Vogler 2001). These waxes coupled with physical structures on the cuticle produce various metallic colors (Pearson et al. 2015).

The head of an adult tiger beetle is the most studied body part because of their two long, thin, 11-segmented antennae, large mandibles, and large compound eyes, in addition to other features (Pearson & Vogler 2001). The antennae are mostly used as sensory organs (Leonard & Bell 1999). Tiger beetle mandibles are not only used for capturing and consuming prey species; males also use their large mandibles to hold onto females during mating and mate guarding. The bulging compact eyes can vary in size depending on the behavior of species. Nocturnal tiger beetles have relatively small and flatter eyes compared to diurnal tiger beetles that have large, bulbous eyes (Leonard & Bell 1999; Pearson et al. 2015).

The tiger beetle thorax is one of the more distinctive among beetles. Its shape, texture, color, and pattern or absence of setae help in identifying different species and genera. Modified forewings, the elytra, cover the soft hind wings and are located on the

top of the abdomen and are the easiest way to identify species. Like many insects, the elytra fold forward revealing the hind wings while in flight and can function as air foils, but they do not flap. The elytra can have various colored micro-sculptures called maculations, which have different shapes among and sometimes within species (Pearson et al. 2015).

The upper surface of the abdomen is almost completely covered by elytra in most species. The lateral and ventral surface of the abdomen is metallic in most species, with color variation between and among species. Like other beetles, the coxae in the hind legs form a ventral plate that is functionally part of the abdominal "armor box". Unlike most insects that have their abdominal spiracles (breathing openings) laterally on the abdomen, abdominal spiracles of tiger beetles are located dorsally underneath the folded hind wings and elytra (Pearson & Vogler 2001).

Cicindelidia haemorrhagica

Cicindelidia haemorrhagica, also known as the wetsalts tiger beetle, is black to brown to dark green from above with three maculations that vary in length but are similar in width with the middle maculation not quite reaching the edge of the elytra. From below, a dark purple or copper is visible with an orange abdomen (Fig 1.1), which is exposed from above when in flight.

Fig 1.1: The orange-red abdomen of *Cicindelidia haemorrhagica*. Photo by M. M. Gotschall.

A unique characteristic associated with *C. haemorrhagica* is that their feces is orange to red. When fleeing from predators, it will defecate as a defense mechanism (Leonard & Bell 1999). Besides YNP, *C. haemorrhagica* occur in various locations in the western United States (Fig 1.2) (Pearson et al. 2015).

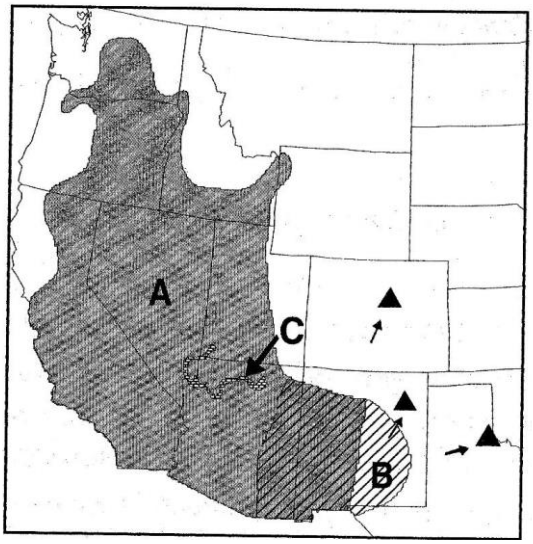

Figure 1.2. Distribution and range of *Cicindelidia haemorrhagica*; **A.** *C. haemorrhagica*; **B.** *C. h. woodgatei*; **C.** *C. h. arizonae*. Reprinted from Pearson et al. (2015).

Where *C. haemorrhagica* occur, they are rarely found far from a water source. These water sources can be ponds, lakes, springs, reservoirs, rivers, irrigated ditches, and oceans. This association of beetles with water is likely associated with predation behavior because *C. haemorrhagica* will feed on concentrated prey items in or along the water's edge. Larvae are found on banks of the water sources that have moist sandy clay edges. Larvae and adults are also found on salt flats, hence the common name wetsalts tiger beetle. Adults are capable of long-distance dispersal although their flights are relatively short. Adults can be active from April to October, although water present and seasonal rainfall can alter this time frame especially in desert areas. June to September is the usual time span for adults of this species. Larvae on the other hand, go through three instars (the phase between two periods of molting) that usually take one to two years before becoming adults.

Both larvae and adults are predaceous, although adults also will feed on recently dead insects caught in thermal pools. Adults engage in a "head-dipping" behavior which is associated with hunting aquatic larvae (for example, ephydrids). Based on these behaviors, the mode of entry for heavy metals is oral and likely associated with eating prey that contain metals. Whether-or-not biomagnification of heavy metals occurs in *C. haemorrhagica* is unclear, but this question should be answered when measurements of heavy metals in prey species are made.

Heavy Metals

Heavy metals are "naturally occurring elements with a high atomic weight and at least five times greater than that of water" (Tchouwuo et al. 2012). Some heavy metals are essential components of specific enzymes, but when any metal occurs at a sufficiently

high concentration, making it poisonous, it produces toxic effects. At these high levels, metals are poisonous and cause harm through various mechanisms such as the production as free radicals, disruption of membrane function, and many others (toxins and poisons are both harmful chemicals at some concentration, however, toxins originate from living tissue while poisons do not). Like other potential toxins, the dose to the heavy metal determines whether the metal causes toxic effects. Organisms cope with poisonous substances by one of three mechanisms: elimination, isolation, or detoxification (Hopkin 1989). Detoxification can occur by chemically breaking down a poisonous substance or adding chemicals to the poisonous substance that interferes with its mode of action. Heavy metals are unique as poisons because (1) as elements, they cannot be chemically broken down to a non-toxic form, and (2) the addition of chemicals to a metal, methyl groups for instance, typically make the metal more poisonous (usually because the addition makes the metal more mobile in an organism). Elements that are essential, such as zinc and iron, have a larger toxic threshold dose than those that are non-essential, making every element's toxic threshold unique to different species. Also, although organic toxins have environmental half-lives mostly based on degradation of the toxin, heavy metals do not degrade. This means that metals released into the environment from mining, industrialization, etc., have long residence times in soils and may be present long after the source is gone (Hopkin 1989). Thus, metal concentrations in soil and water determine, in part, the bioavailability of a metal in an environment. Bioavailability of heavy metals also depends on temperature, pH, and metal species (metal species are "toxic metals, metalloid compounds, and metal-based drugs …with endogenous ligands" Gailer 2013).

A metal's toxicity to plants, vertebrates, and invertebrates depends on its form which can be separated into three categories : simple aquated (bound to water) metal ions (e.g. $Fe(H_2O)^{3+}_6$), metal ions complexed by inorganic anions (e.g. $CuCl^+$) or metal ions complexed by organic ligands (e.g. amino acids such as $Cu(NH_2CH_2COO)_2$) (Jorgensen & Jensen 1984). Environmental pollutants of most concern are group B and borderline metals (Table 1.1) that form inorganic complexes in saline solutions and are very lipid soluble (Hopkin 1989). Likewise, Simkiss (1983) showed that group B metals, such as copper, cadmium, and mercury, form inorganic complexes in saline solution and are lipid soluble causing a direct pathway across the microvillus boarder of the gut in terrestrial invertebrates.

Table 1.1. Some essential and non-essential metal ions of importance as pollutants: class A (oxygen-seeking), class B (sulphur- or nitrogen-seeking), and borderline elements based on the classification scheme of Nieboer & Richardson (1980).

Class A	**Borderline**	**Class B**
Calcium	Zinc	Cadmium
Magnesium	Lead	Copper
Manganese	Iron	Mercury
Potassium	Chromium	Silver
Strontium	Cobalt	
Sodium	Nickel	
	Arsenic	
	Vanadium	

Cicindelidia haemorrhagica in Yellowstone National Park

Cicindelidia haemorrhagica have been recorded in YNP since 1891 (Hubbard 1891). Hubbard (1891) noted that adults were walking on the water at Mammoth Hot Spring, YNP, but he did not discuss much about their ecology nor distribution within YNP. While visiting in 2006, L. G. Higley saw adults not only around the thermal feature but running through the water, eating prey species, and holding a water droplet under the abdomen (Fig. 1.3).

Figure 1.3. Photograph taken of *Cicindelidia haemorrhagica* in Mammoth Hot Springs, Yellowstone National Park, during the summer of 2006 by L.G. Higley.

This observation led to further investigation in 2016 – 2018 by Willemssens (2019), with the objective of exploring how these tiger beetles are able to survive in thermal springs that exceeded known temperature tolerances of insects, as well as high and low pH, and heavy metals. Adults and larvae were found throughout YNP, but they were strictly associated with the thermal springs in the area. Heavy metal concentrations varied from each site and temperatures ranged from 29.1 ˚C to 70 ˚C, which can affect toxicity of the heavy metals (Hopkin 1989) and had pH values ranging from 2.73 to 14 (Willemssens 2019).

Along the water's edges, there are red, brown, and green structures on the soil and water that are algal and bacterial mats. *Cicindelidia haemorrhagica* scavenge and hunt on these mats, which are known to have ephydrid larvae living within and ephydrid adults nearby. Tiger beetles are known to feed on ephydrids living in algal mats (Zack 1983). *Cicindelidia haemorrhagica* were also observed feeding on various other insects that had fallen into the thermal water and died due to unknown factors (likely extreme heat)

(Willemssens 2019). Because of living in a thermal feature, the likelihood of a lower mortality rate due to over wintering is possible, but because of the environmental extremes, it is unknown if the adults burrow to over winter, or if they remain active (Willemssens 2019). If adults over winter in YNP, it could contribute to the need for metal sequestration or elimination.

Willemssens (2019) examined the differences in diurnal behavior between YNP adults and those at a salt flat in Idaho. Within YNP, *C. haemorrhagica* seemed to be more opportunistic predators than those observed in Idaho, because the Idaho population does not have as many limitations, resulting in a larger variety of prey capture, as well as incidents of adult cannibalism. Regarding adaptation to environmental conditions, YNP adults exhibited limited thermoregulatory behavior and that behavior was not directly correlated to cooling rather warming in the morning. Willemssens (2019) observed adult *C. haemorrhagica* in YNP in water at temperatures as high as 70 ˚C, with demonstrating few thermoregulatory behaviors. In contrast, she observed *C. haemorrhagica* in Idaho at water temperatures of 30.34 ˚C (± 5.51), and the beetles engaged in thermoregulatory behavior such as moving into shade, stilting, and abdomen-dipping in water. Willemssens (2019) concluded that YNP adults may be thermophilic (heat loving) rather than thermotolerant, because YNP adults have a close association with hot thermal pools (greater than 40 ˚C) and exhibit limited thermoregulatory cooling behaviors, which also supports terming them extremophiles.

Heavy Metals within Yellowstone National Park

Heavy metals have always been associated with YNP given its volcanic origin and many geothermal features. Geysers, fumaroles, vents, and hot springs emit gaseous forms of mercury, sulfur, and other heavy metals. This makes the geothermal features natural sources for these elements in the atmosphere (Bennett & Wetmore 1999). Warnings are posted about the levels of heavy metals, bacteria, and other contaminants in various areas throughout YNP, so that visitors do not accidentally poison themselves or others. The U. S. National Park Service recognizes arsenic, fluoride, sodium, sulfate, lead, copper, chloroform, selenium, nitrite, nitrate, mercury, dichloroacetic acid, etc. in Old Faithful alone (National Park Service 2019). This site is relatively close to Rabbit Creek, which is one of my research sites.

Studies have been done on toxic metal levels in megafauna in YNP based on their feces (Chaffee et al. 2007). While examining elk, moose, bison, and grizzly bears, researchers found poisonous metals for vertebrate species (e.g. As, B, Be, Ce, Cl, Cs, F, Hg, K, Li, Mo, Rb, S, Sb, Si, and W) in their feces that came close or exceeded normal thresholds for those vertebrate species. However, while these animals should have been experiencing heavy-metal poisoning, they were relatively healthy (Chaffee et al. 2007). These types of metals are commonly enriched in thermal waters in the YNP area, rocks altered by these waters, sinter and travertine deposits, and soils and stream sediments derived from the rocks. Some of these elements, such as As, F, Hg, and Mo, can be toxic to wildlife and be magnified in the food chain (Chaffee et al. 2007). For example, mercury has been studied and monitored within fish in YNP. Many people fly fish in the

area and there is a concern about whether it is safe to eat fish caught in YNP (National Park Service 2018).

A common poisonous metal element in YNP's environment is arsenic. It also ranks first on the Superfund list of hazardous substances and is of concern as a "universal" poison because few species are immune to arsenic toxicity. Arsenic has been studied within YNP in relation to algal mats found in thermal springs. Studies with the alga *Cyanidioschyzon* spp. show arsenic detoxification characteristics that can lead to wider impacts (Qin et al. 2009).

Heavy Metals in Terrestrial Invertebrates

Because of selective pressures in the marine environment, terrestrial organisms may have been constrained by biochemistry and internal anatomy to adapt strategies for metal regulation (Dallinger & Rainbow 1993). Although terrestrial invertebrates may have had constraints, some species evolved different ways in accumulating and eliminating metals with variations even in closely related species and subspecies that may live in the same environment (Dallinger & Rainbow 1993). Despite species-specific variance, accumulating specific metals within specific tissues seems to be a uniform technique of some invertebrates (Hopkin 1989). Strategies for metal regulation can be affected by their food, the structure and function of the digestive system, and other necessary functions such as molting their exoskeleton (Hopkin 1989).

Dallinger & Rainbow (1993) reviewed how terrestrial invertebrates have evolved to avoid heavy metal toxicity. Although excreting unwanted essential and non-essential metals through ultrafiltration or active transport mechanisms is how most vertebrates

avoid toxicity, terrestrial invertebrates are more at risk of dehydration, so the release of excretory fluids are restricted. Apparently, this has led to the evolution of toxin removal through defecation or a storage of the metals. Limitations to remove metal toxins can pose a large risk for invertebrate species, and many have had to adapt to inactivate and retain metals by intercellular compartmentalizing. These mechanisms have been found in nematodes, gastropods, arachnids, insects, and other invertebrate taxa.

The exchange of metals in aquatic invertebrates is typically associated with the liquid medium, but in terrestrial invertebrates it is the epithelium of the gut that is assumed to be responsible for the exchange of metals with the environment. This is because the gut epithelium plays a large role in most terrestrial invertebrates' nutritional physiology (Dallinger & Rainbow 1993; Hopkin 1989). The epithelium is usually only a single cell thick and acts as a barrier between the lumen containing food, and the internal environment. Therefore, the gut is directly involved with uptake, transportation, storage, and excretion of metals (Hopkin 1989). For example, Dallinger & Rainbow (1993) explain that high levels of copper, zinc, cadmium, and lead have been recorded in metal-exposed species in polluted environments and in laboratory settings in the midgut glands.

Metals accumulated in some organisms are not ordinarily distributed evenly throughout a body, indicating that only some organs and tissues aid in metal accumulation while others do not (Dallinger & Rainbow 1993). Certain metals can be moved into specific cells that are then sequestered by vesicular compartments. These are described as "concretions" or "metal-concretions" in intestinal cells of insects (Dallinger & Rainbow 1993). Essential and non-essential metals in the midgut of terrestrial

invertebrates that are in excess, are stored in metal-binding proteins and different types of membrane-bound granules. The metal-containing granules are lysosomal residual bodies which are insoluble and do not seem to breakdown intercellularly which forms a cellular storage-detoxification system (Dallinger & Rainbow 1993; Hopkin 1989). In energy terms, permanent storage of these granules would be the least expensive, as long as they do not occupy a sufficiently large volume within the cells that they interfered with normal cytological functions. Non-essential elements can be inactivated and detoxified or stored and remobilized by sequestration within vesicles.

However, many terrestrial invertebrates can eliminate toxic metals by cellular processes. These can include the removal of degenerated cells, exocytosis, or extrusion of metals-containing vesicles or granules into the lumen of the digestive tract, but variations are considerable between invertebrate taxa (Dallinger & Rainbow 1993). In spiders and centipedes, metal-containing granules spill out into the lumen at the end of each digestive cycle, but also occur during molting. If the granules are stored in the hindgut of an arthropod, they can only be excreted through the breakdown of the epithelium during molting (Dallinger & Rainbow 1993; Hopkin 1989).

Although it is assumed that elements deposited in the exoskeleton is a way for terrestrial arthropods to eliminate unwanted metals, this assumption remains unproven (Hopkin 1989). Within most insects, metals are accumulated in distinct sections of the alimentary canal such as the Malphighian tubules (Dallinger & Rainbow 1993). In eight different species of spiders, metals within the cuticle are present as a surface deposit (Clausen 1984). Also, metals ‘in’ the cuticle can be associated in other tissues as in

Collembola which void the gut epithelium at each molt. Likewise, *Lithobius variegatus* (a centipede species) had a body wall rich in zinc after molting while the molted exoskeleton did not contain zinc, resulting in the conclusion that the metal within the body wall was associated with other tissues bound to the cuticle (Hopkin & Martin 1984).

Metal-binding proteins, such as cytosolic metallothioneins, are also used to store and eliminate non-essential metals. These proteins could have an important biological function due to it sequestering and, in turn, deactivating toxic metals in the cytoplasm of invertebrate cells. However, it may also be a multifunctional protein, and detoxification of non-essential metals is one of several functions it performs. Dallinger & Rainbow (1993) discussed how because of the duplication if gene encoding metallothioneins with cadmium, some populations of *Drosophila melanogaster* have been able to increase cadmium tolerance. Still, there are many questions around metallothioneins and not only what they are capable of, but what they can do in terms of building tolerance and/or resistance.

Temperature is the main abiotic factor that affects metal assimilation (Hopkin 1989). A higher temperature means a greater rate of food consumption resulting in food spending less time in digestive enzymes. Therefore, invertebrates consuming food at lower temperatures are subject to higher metal accumulation than if at a higher temperature. Rapid temperature fluctuations can also have an impact on terrestrial invertebrates in the field as well as day length and food availability (Hopkin 1989).

Most toxic metal studies with insects have focused on pollutants and pesticides, and are laboratory-based, emphasizing on growth and reproduction. Because insects have

a smaller midgut, there are only a few examples of insects that accumulate metals to the same extent as other terrestrial invertebrates such as isopods and earthworms. Likewise, they do not need to have adaptations their counterparts have because they do not live long enough for the metal toxicity to occur (Hopkin 1989).

According to Salmons and Stigliana (1995), soil organisms can be exposed to pollutants by ingestion or oral uptake of soil particles, dermal uptake of pollutants from the soil pore water and possibly by exposure of pollutants in the soils' air phase. Organisms with exoskeletons would be less likely to uptake heavy metals directly from the soil but more so from oral uptake. On the other hand, soft-body terrestrial invertebrates would mainly be exposed to the heavy metals through soil pore water which would include larvae (Salmons & Stigliana 1995).

Field measurements of heavy metal concentrations among insect species are sparse. In one example, Warnick & Bell (1969), found that in heavy metal contaminated water, arsenic concentrations in mayfly nymphs exceeded 14 mg/L (of nymph) and in damselfly nymphs exceeded 20 mg/L (of nymph). Field concentrations of lead have been recorded from various whole beetles at concentrations greater than 20 μg/g (of beetle) close to industrial contamination sources (Hopkin 1989). Higher concentrations of other metals have been found in beetles at uncontaminated sites, specifically 100 – 300 μg/g (dry weight of beetle) of zinc and 15 – 50 μg/g (dry weight of beetle) (Carter 1983). Carter also noted that much of the zinc was present in the mandibles of herbivorous species, representing over 1% of mandible mass.

Ashbaugh (2018) examined two species (*Cicindela circumpicta* and *Cicindela togata*) as a main food source for snowy plovers (*Charadrius nivosus*) in conjunction with being biological indicators for metal accumulation. He found evidence of bioaccumulation of selenium and arsenic. Selenium was measured at 5.93 ppm (mg/L of beetle wet weight) (*C. togata*) and 6.20 ppm (mg/L of beetle wet weight) (*C. circumpicta*) and arsenic ranged from 1.58 – 2.66 ppm (mg/L of beetle wet weight) (*C. togata* and *C. circumpicta*).

Thesis Observations and Hypothesis

The objectives of this study are to

1. Examine variations in heavy metal concentrations in mandibles, elytra, abdominal venter, soft tissue viscera, and the rest of the exoskeleton from different YNP locations and Idaho
2. Evaluate potential mechanisms for transfer of metals to tissues in *C. haemorrhagica*

My hypotheses is that *Cicindelidia haemorrhagica* located in Yellowstone National Park are bioaccumulating heavy metals and that concentrations and distributions of heavy metals in *C. haemorrhagica* differ between populations in YNP and a known population outside of YNP not associated with a thermal spring in Idaho.

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CHAPTER TWO

BIOACCUMULATION AND PARITIONING OF HEAVY METALS IN CICINDELIDIA HAEMORRHAGICA IN YELLOWSTONE NATIONAL PARK

By Monica M. Gotschall

Advisor: Leon G. Higley

Introduction

Yellowstone National Park (YNP) is known for its geysers, thermal features, and populations of native animals, including bison, elk, and bears. The thermal areas are manifestations of volcanic activity at the surface of the massive (45 – 70 km diameter) caldera that forms most of YNP. Yellowstone has the largest occurrence of thermal areas on earth, which include mud pots, fumaroles, hot springs, and geysers. The thermal areas have fragile soils sometimes with underlining vents and openings. Features within the thermal areas, can produce high temperature steam, superheated water, and multiple heavy metal contaminants. For instance, hot spring water temperatures typically range from 40 – 100 ˚C, vary in pH between 0 – 14, and may have heavy metals ("naturally occurring elements with a high atomic weight and at least 5 times greater than that of water") (Tchouwuo et al. 2012), including arsenic, mercury, copper, and lead (Inskeep & McDermott 2004; Planer-Friedrich et al. 2006; Yellowstone National Park 2019).

Because thermal areas have temperatures and chemical properties that are hostile to most organisms, these are regarded as extreme environments and the organisms that can live there are called extremophiles. For example, few organisms can tolerate water temperatures above 45 ˚C, highly acidic (pH <3.0), or highly basic (pH >9.0) soil and waters (Gupta 2014), which occur at many sites in YNP. Archaea, bacteria, and algae

associated which are associated with Yellowstone's hot springs are the extremophiles that are most studied, but there are other types of extremophiles.

Perhaps the most diverse group of animal extremophiles at Yellowstone are insects, specifically species of flies, true bugs, and beetles. *Cicindelidia haemorrhagica*, is one of these insects that live exclusively in the thermal features of YNP (Fig. 2.1). They were first reported in YNP in 1891 by Hubbard but were not studied in this habitat until recently. In 2006, L. G. Higley observed beetles in Mammoth Hot Springs, YNP (the same feature where Hubbard first noticed the beetles!) and noted that the beetles did not seem to engage in behavioral cooling, which tiger beetles typically do when in hot environments (Fig. 1.2). Ultimately, this led to cooperative research between the Higley lab at the University of Nebraska-Lincoln, the Peterson lab at Montana State University, and scientists in the National Park Service at YNP. Willemssens (2019) presented some of the first results of this research collaboration.

Fig. 2.1. *Cicindelidia haemorrhagica* at Rabbit Creek, Yellowstone National Park. Photo by M. M. Gotschall.

Being approximately 10 – 14 mm long and having a bright orange abdomen, *C. haemorrhagica* is thought to be the apex invertebrate predator within the thermal features (Pearson et al. 2015; Willemssens 2019). Until 2016, there was little known about its distribution in YNP. Willemssens (2019) observed its distribution and discovered that they were strictly associated with thermal springs and appeared to live out their life cycles in this environment. In 2017, the McDermott, Peterson, and Higley labs examined potential bioaccumulation ("bioaccumulation occurs when the compound concentrates in living organisms or tissues") (Kodavanti et al. 2014) of heavy metals in adults, and obtained preliminary evidence that bioaccumulation was occurring.

In this research, I measure bioaccumulation of heavy metals in *C. haemorrhagica*, and dissect adults into different segments to find heavy metal concentrations and levels to determine where in the beetle bioaccumulating might occur. My hypotheses is that *C. haemorrhagica* located in YNP are bioaccumulating heavy metals and that concentrations and distributions of heavy metals in *C. haemorrhagica* differ between populations in YNP and a population outside YNP in Idaho.

Materials and Methods

Site Descriptions

We selected study sites within YNP based on beetle abundance, accessibility, previous research, limited or no public view (a NPS requirement), and formal YNP approval. The sites chosen were Dragon Hot Spring (Fig. 2.2) (2018 – 2020) and Rabbit Creek (Fig. 2.3) (2019 – 2020) under National Park Service Yellowstone National Park Research Permit #8100 and #7092. Dragon Hot Spring is a relatively well-studied acid-

sulfate-chloride spring that is home to "*Hydrogenobaculum*- like organism" (D'Imperio et al. 2007) and thermophilic chemolithotroph species with high levels of As(III) (33µM) and $MeHg^{+}$ (4 – 7 ng/g dry weight of mat biomass) in the source water located in the Norris Geyser Basin (Boyd et al. 2009; D'Imperio et al. 2007; Fouke 2011; Inskeep et al. 2013). The pH was 2.88 (± 0.17) in 2018 with water temperatures reaching 68.5 ˚C (Willemssens 2019). However, in some areas, running water within the site has sufficiently lower temperatures (49.31 ˚C ± 4.42) (Willemssens 2019) to allow growth of acidophilic *Zygogonium* spp. in mats that can accommodate stratiomyid and ephydrid populations (Boyd et al. 2009; D'Imperio et al. 2007, Zack 1983).

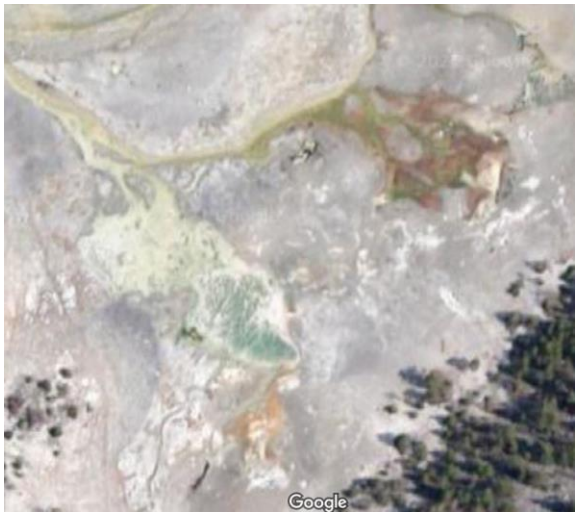

Figure 2.2. Dragon Hot Spring, Yellowstone National Park and surrounding area in the Norris Geyser Basin where *Cicindelidia haemorrhagica* were collected. Google maps imaging on 19 April 2021.

Located in the Midway Geyser Basin with many alkaline thermal water sources is Rabbit Creek. With its wide range of water temperatures, Rabbit Creek can accommodate many different communities of acidobacteria and cyanobacterial mats (Weltzer & Miller 2013; Miller et al. 2009). Rabbit Creek was the largest site with a steep thermal gradient

throughout the entire stream, with estimated changes in water temperature of 7.5 ˚C for every 100 m of continuous waterway in some diverged waterflow locations. This gradient was determined by the level of stream flow and the injection of water from additional heated water sources that increased the overall temperature of the creek (J. L. Bowley, unpublished). The main water flow originates from one alkaline hot spring at the eastern most point and contains deposits of alkaline siliceous rock throughout most of the water's edge and creek bed (J. L. Bowley, unpublished). Eventually, the water flows from the head spring westward into the Firehole River (Miller et al. 2009). Rabbit Creek's pH was 9.88 in 2019, which indicated that *C. haemorrhagica* has some pH tolerance for the water in which they hunt for food in (Willemssens 2019). Target field research sites for Rabbit Creek were initially selected based purely on beetle presence while walking along the embankments of the creek. After initial scouting was finished, the decision was made to use a median within the creek we called "The Island" (Fig. 2.2) based on ease of access, observable beetle food sources, overall beetle abundance, water temperatures, and water pH. There was also a water source that was continuously flowing, year around, into the creek that was directly underneath "The Island", heating the underside of the exposed land through conduction and releasing water of an adequate temperature to facilitate microbial growth which leads to the production of cyanobacterial mats (J. L. Bowley, unpublished).

Figure 2.3. Yellowstone National Park site, Rabbit Creek, showing “The Island” where collection of *Cicindelidia haemorrhagica* was conducted. Google maps imaging on 19 April 2021.

Cicindelidia haemorrhagica’s population in Idaho, was selected because it was a known habitat for the beetles and is not associated with a hot spring. This site had water temperatures of about 30 ˚C and pH measurements of approximately 8.22 and the *C. haemorrhagica* population resides on a salt flat immediately adjacent to the Snake River near Mountain Home, Idaho (42°56'06.5"N 115°45'00.9"W) (Willemssens 2019) (Fig 2.4). *Cicindelidia haemorrhagica* share this habitat with a much less abundant *C. punctulata* and seem to be the only tiger beetle species that consistently occupies the salt flat throughout days with full sun (Gotschall et al. in review). The presence of *C. haemorrhagica* was recorded in 2016 by J. Runyon (USFS), and Idaho Fish and Game (IDFG) gave permission to enter the area and collect samples as needed.

The three field research sites were used for sampling *C. haemorrhagica*, water, and soil.

Fig. 2.4. Our third research site outside of Yellowstone National Park where we collected *Cicindelidia haemorrhagica* on a non-hot spring salt flat near Mountain Home, Idaho, 42°56'06.5"N 115°45'00.9"W. Photo by Bowley, J. L., unpublished.

Tiger beetle collection

Cicindelidia haemorrhagica adults were collected using a heavy duty sweep net with a 38 cm diameter and placed in 20 ml glass collection vials with small holes in the lid for ventilation (1 beetle per vial). The vials were then placed in a portable Yeti Hopper M30 Soft Cooler until they were brought back to the Peterson lab at MSU within 24 hours of collection. The beetles were collected between 15:00 and 19:00 in July of years 2018 – 2020. In the laboratory, each vial's lid was replaced with an airtight lid and set in the freezer at -20 ˚C.

In a separate vial, individuals were dipped into 10 ml of dichloromethane 3 times for 10 seconds. Dichloromethane was used to remove cuticular waxes from *C.*

haemorrhagica for separate metal analysis. The beetles then were dissected into five sections: mandibles, elytra, viscera (abdominal soft tissues), abdominal venter, and the rest of the exoskeleton, each placed in separate vials. Each section was weighed and recorded to calculate accurate metal concentrations.

Water collection

Water samples were taken in 2018 and 2019, however, Idaho samples were not viable. At each YNP site, three, 50 ml water samples were collected and placed in 50 ml falcon tubes with 2.5 ml of HCl. Water samples were stored and transported to the Peterson lab as described for beetles, however, at the laboratory water samples were refrigerated rather than frozen.

ICP-MS Preparations

Specifications: Total metals were measured at the Montana State University Proteomics, Metabolomics, and Mass Spectrometry Facility on an Agilent 7800 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) with reaction cell (H_2 and He mode capability). A certified standard from CPI International (Product # 440 – 121116NC02) was used to generate calibration curves and check standards for quantification of trace metals. Their ICP-MS used a quadrupole mass spectrometer with an electron multiplier detector for analysis. The lower quantification limit and upper quantification limit a for all metals I am reporting (arsenic, copper, lead, cadmium, and selenium) are 0.5 ppb and 500 ppb. Blanks and check standards throughout the runs to ensure calibrations were correct.

Tiger Beetles

Beetle processing for elemental analysis was modified from procedures used in the McDermott lab at MSU (McDermott, unpublished.). Acid (2 ml of concentrated HCl and 2 ml of concentrated HNO_3) was added to each vial (containing either mandibles, elytra, abdominal venter, viscera, or other tissues from one beetle) (Fig. 2.5 A – B), placed in an 8 quart Insta Pot® Ultra10-in-1 Multi-Use Programmable Cooker (Model Ultra 80), and heated to dissolve beetle tissues. The Insta Pot® Ultra was set to custom settings with the heat at 97.78˚ C with no pressure, and a time of 25 minutes (Fig. 2.5 C). Sets of 18 samples were processed in the Insta Pot® Ultra in 3 metal cooling racks with 250 ml of distilled water (Fig. 2.5 D). After heating, samples remained in the Insta Pot® Ultra for one minute, and then were removed to cool for one hour (Fig. 2.5 E. This procedure helped ensure the chemical reaction of HCl and HNO_3 was slowed before further processing.

With the samples dissolved in the acids, 16 ml of distilled water was added to each vial to total 20 ml for each sample (Fig. 2.5 F). Then, 8 ml of each sample was placed in 15-ml conical Falcon tubes for analysis by the ICP-MS for a total metal analysis. This instrument provided measurements for total metal analysis, but for the sake of this study we observed arsenic, cadmium, copper, lead, and selenium, however, some heavy metals (antimony and mercury) could not be determined because appropriate detectors were unavailable. It should be noted that samples were analyzed with a new ICP-MS instrument installed in 2020 (2018 and 2019 samples were not processed until

2020), and 2019 samples were among the first samples processed with this new instrument.

Cuticular waxes were processed with the same procedures as beetles with one modification. Dichloromethane was evaporated (through negative pressure in a fume hood) before acids were added. Removal of dichloromethane was necessary because it might otherwise be damaging to the ICP-MS.

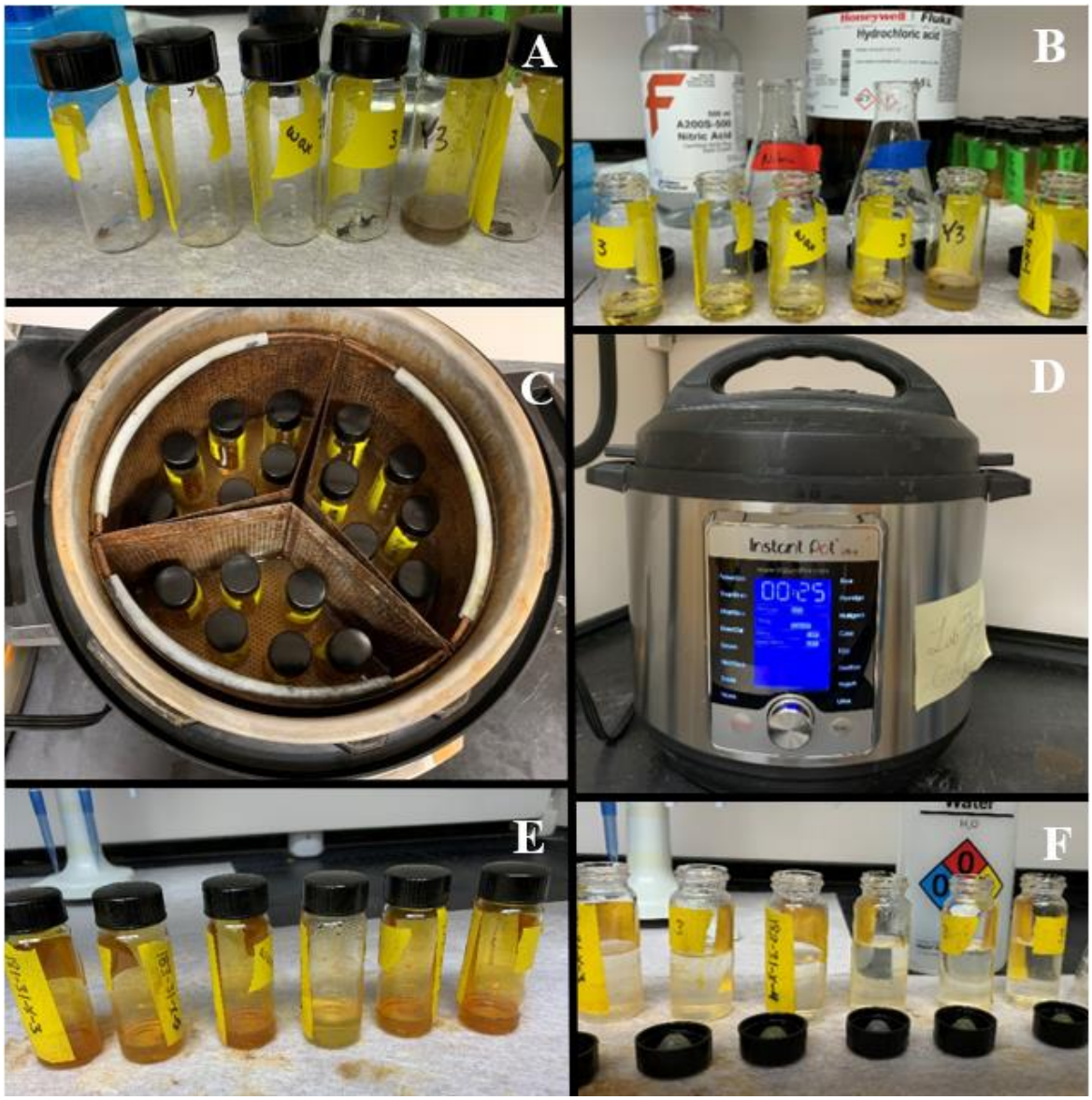

Fig. 2.5. Preparation of *Cicindelidea haemorrhagica* using an 8-quart Insta Pot® Ultra10-in-1 Multi-Use Programmable Cooker (Ultra 80) for metal analysis at Montana State University's Proteomics, Metabolomics, and Mass Spectrometry Facility, with an Agilent 7800 Inductively Coupled Plasma Mass Spectrometer (ICP-MS). A: Each dissected segment (mandible, viscera, elytra, other exoskeletal tissues, and waxes) of *C. haemorrhagica* in an individual glass vial. B: Two ml of HCl (concentrated) and two ml of NHO_3 (concentrated) added to each vial. C: Eighteen samples were processed at a time using three metal cooling racks in 250 ml of distilled water. D: The Insta Pot® Ultra was set to custom settings, at 97.78 ˚C for 25 minutes under no pressure. E: Samples were set aside to cool for 1 hour so the acidic reaction could decelerate before opening the vials. F: Sixteen ml of distilled water was added to each vial to total 20 ml. Eight ml of homogenized mixer was then be taken to be processed through the ICP-MS.

Water

Once water samples were in the laboratory, 20 ml of each were placed into separate vials and 1 ml of HNO_3 was added to oxidize metals for analysis. Samples were diluted 1:10 (2 ml of each sample was taken out and placed in a new vial and 18 ml of distilled water was added), because undiluted samples were above qualifying limits of the ICP-MS.

Data analysis

Because so few individual beetles were available for analysis per site (four per site, per year), we were concerned that high variability might skew results. Additionally, in dissecting individuals to examine metal concentrations in different body parts (specifically cuticular waxes, viscera [non-exoskeletal tissues], and exoskeleton [mandibles, elytra, venter, and other exoskeleton]), we recognized that we might not be able to exclude all musculature and connective tissue from exoskeletal tissues. Consequently, we tested all calculated heavy metal weights and concentrations with interquartile range (IQR) tests to exclude outliers.

Data from viscera and other tissues varied by as much as three orders of magnitude within the same location, and because of this extreme variation were not included in the analysis. Also, because we only had water concentrations from 2018 and 2019 from YNP, these are not reported here.

Application of IQR tests by year and element was conducted in Excel. All other statistical tests were conducted in SAS academic edition (SAS Institute Inc. 2015. SAS/IML® 14.1 User's Guide. Cary, NC: SAS Institute Inc.) The influence of year, site, and their interactions were examined by element using Proc Mixed procedures. We also

examined differences between Idaho and YNP beetle populations by averaging data from the Dragon Hot Spring and Rabbit Creek sites in YNP for comparison with Idaho data. Finally, some tissues (venter) were not dissected in all years, and the Rabbit Creek site was not sampled in 2018 (as indicated in the Results).

Results

The mean weights and concentrations of metals found in *C. haemorrhagica* are reported in Fig. 2.6, and data by and across years are reported in Fig. 2.7.

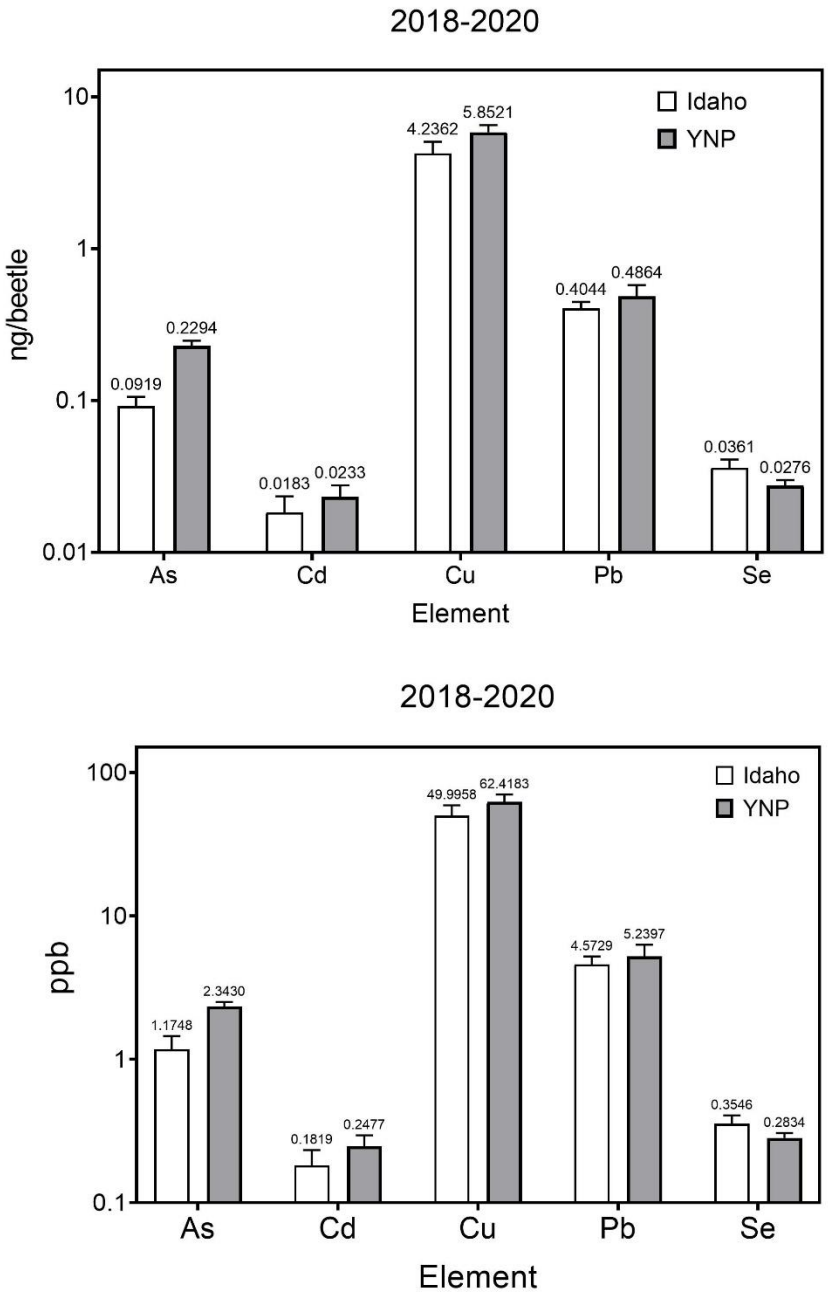

Figure 2.6. Mean weights (ng/beetle) and mean total concentrations (ppb = ng/ml) per beetle (wet weight) of heavy metal contaminants in *Cindelidia haemorrhagica* from Idaho (Loveridge site) and Yellowstone National Park (YNP, average of Dragon Hot Springs and Rabbit Creek) populations in 2018 – 2020. Means and standard errors based on n=4 per site, see methods for more details.

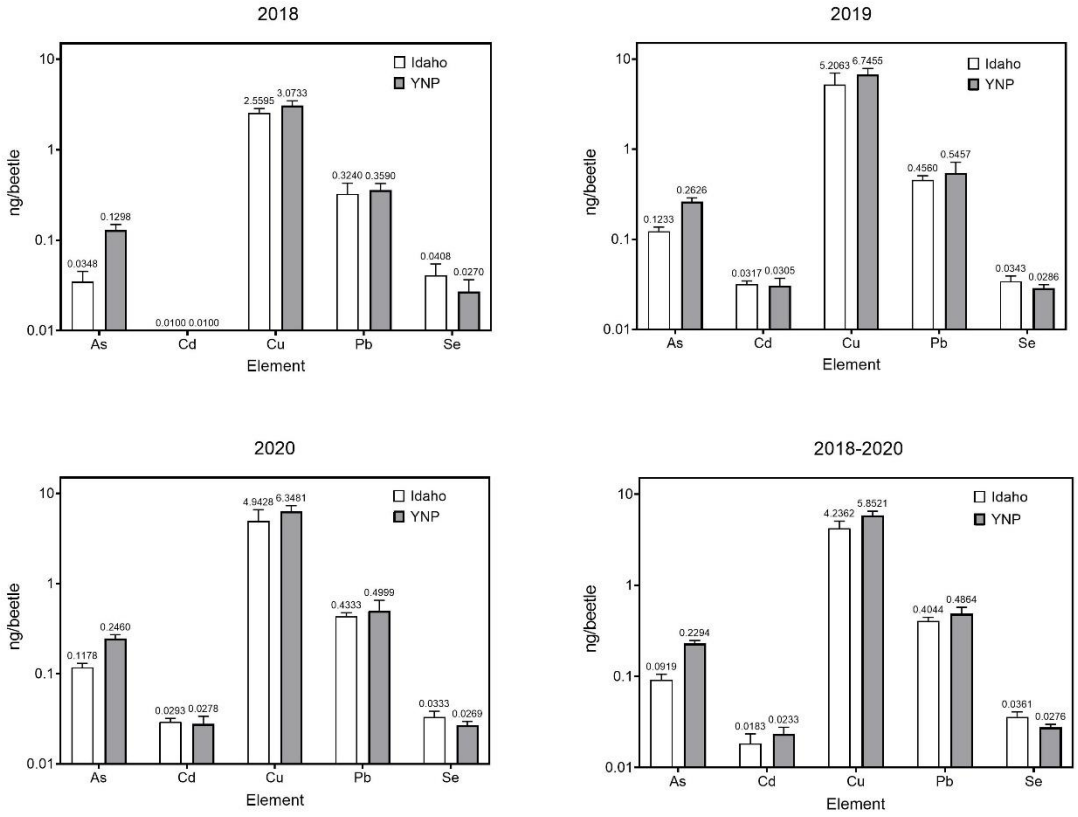

Figure 2.7. Mean weights (ng/beetle) of heavy metal contaminants in *Cicindelidia haemorrhagica* from Idaho (Loveridge site) and Yellowstone National Park (YNP, average of Dragon Hot Springs and Rabbit Creek) populations by and across years 2018–2020. Means and standard errors based on n=4 per site, see methods for more details.

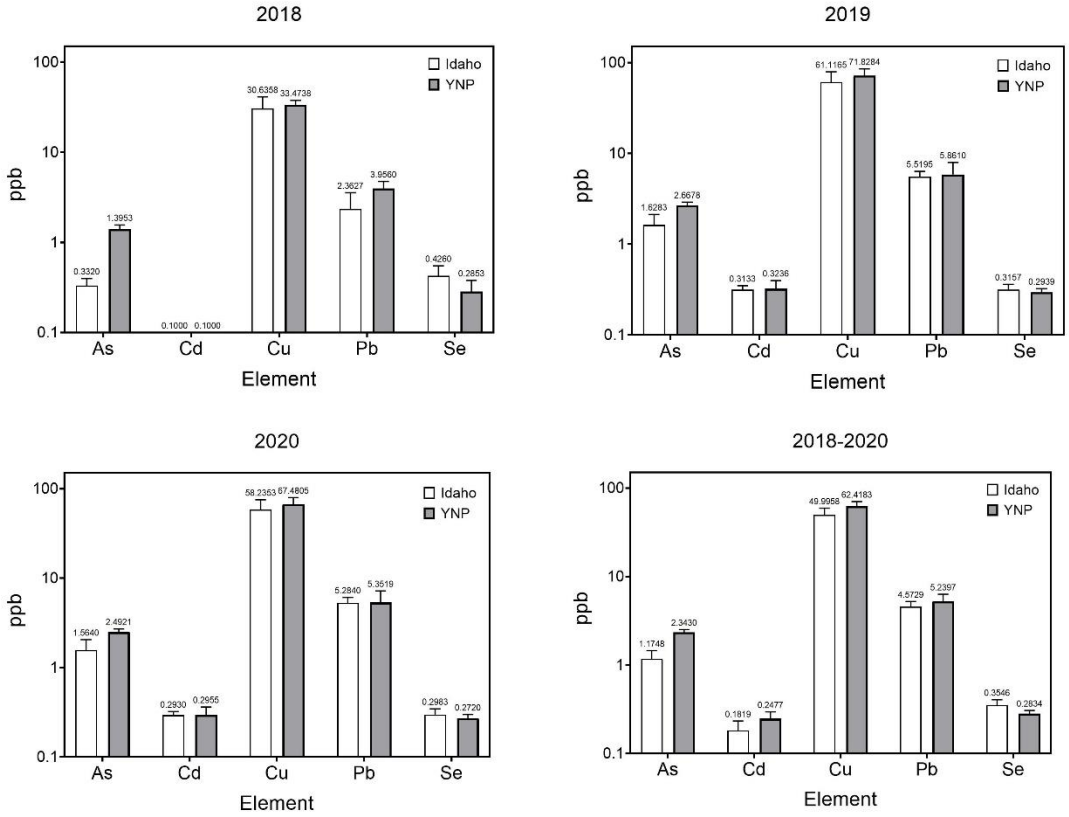

Figure 2.8. Mean concentrations (ppb = ng/ml) per beetle (wet weight) of heavy metal contaminants in *Cindelidia haemorrhagica* from Idaho (Loveridge site) and Yellowstone National Park (YNP, average of Dragon Hot Springs and Rabbit Creek) by and across years 2018 – 2020. Means and standard errors based on n=4 per site, see methods for more details.

Table 2.1. Main effects of year, location (Idaho vs. YNP) by mixed model analysis of total weight for As, Cd, Cu, Pb, Se (only significant effects are shown). "Idaho" represents the Loveridge site and "YNP" represents the average of Dragon Hot Springs and Rabbit Creek. Means and standard errors based on n=4 per site (data from Dragon Hot Springs and Rabbit Creek were averaged before analysis, therefore, n=4 for YNP), see methods for more details.

Element	Main effect	Num DF	Den DF	F Value	Pr > F
As	year	2	26	17.99	<0.0001
	YNP	1	26	22.89	<0.0001
	year*YNP	2	26	3.33	0.0514
Cd	year	2	26	39.76	<0.0001
Cu	year	2	24	5.14	0.0139
Pb	year	2	22	6.3	0.0068
Se	YNP	1	26	5.64	0.0253

Unfortunately, because detectors were unavailable for all metals of interest on the ICP-MS, we were unable to determine weights or concentrations of antimony or mercury. All metals occurred in concentrations of ppb (ng/ml) in all years.

Statistical comparisons of Idaho vs. YNP by total weight and concentration are presented in Table 2.1. Both by weight and concentration, arsenic levels were higher in YNP than the Idaho site, as anticipated. Other metals were not different except for selenium, which was greater in the Idaho site, although selenium concentrations were low at all sites.

Although differences in metal availability can differ by year at YNP based on changes in geothermal activity, this does not explain the significant differences in metal amounts or concentrations across all metals (Fig. 2.7 and 2.8, Tables 2.1 and 2.2). By t-tests of LS means for each metal, 2019 was different from other years in all instances (see appendices for specifics). Measurements of metals in 2019 were as much as 10 times greater than in 2018 and 2020, and because increases associated with 2019 occurred in samples from all sites (including Idaho), this variation is not geothermal. Here, I report averages across years as the best estimates available and show data by year to illustration variation. Moving forward, measurements of metal contamination with simultaneous calibration standards are needed.

Table 2.2. Main effects of year, location (Idaho vs. YNP) by mixed model analysis of total concentration for As, Cd, Cu, Pb, Se (only significant effects are shown). "Idaho" represents the Loveridge site and "YNP" represents the average of Dragon Hot Springs and Rabbit Creek. Means and standard errors based on n=4 per site (data from Dragon Hot Springs and Rabbit Creek were averaged before analysis, therefore, n=4 for YNP), see methods for more details.

Element	Main effect	Num DF	Den DF	F Value	Pr > F
As	year	2	25	9.65	0.0008
	YNP	1	25	10.27	0.0037
Cd	year	2	25	25.43	<0.0001
Cu	year	2	26	7.26	0.0031
Pb	year	2	23	7	0.0042
Se	year	2	24	2.55	0.0989
	YNP	1	24	5.1	0.0334

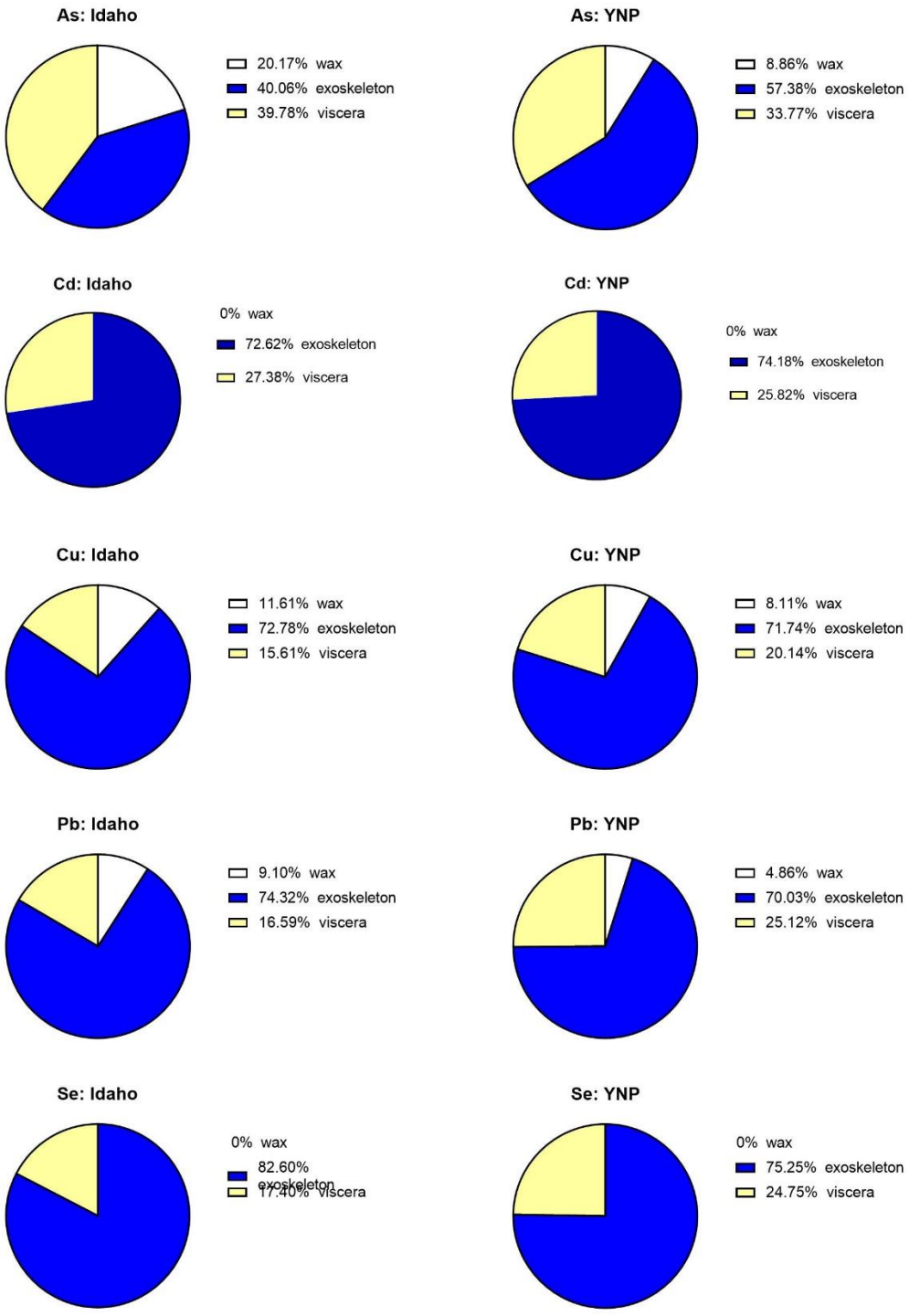

Figure 2.9. Percent distribution by weight of heavy metal contaminants in cuticular wax, exoskeleton, and viscera (non-exoskeletal tissues) of *Cindelidia haemorrhagica* in Idaho and Yellowstone National Park (YNP) populations averaged for 2018 – 2020.

The percent distribution of metals in beetle cuticular waxes, exoskeleton, and other tissues are illustrated for Idaho and combined YNP populations in Fig. 2.9. With all metals other than copper, a greater proportion of metal accumulation occurred in the exoskeleton of Idaho beetles than in YNP beetles. By weight and concentration in exoskeleton (Tables 2.3 and 2.4), arsenic and selenium had statistically greater accumulations in Idaho than in YNP populations.

These results strongly indicate differences in the physiology of arsenic and selenium mobilization between Idaho and YNP beetles. This difference could be associated with dose, that is, the levels of arsenic and selenium, or with genetic differences between populations. Accumulations of arsenic in YNP populations were ca. 3x those of Idaho populations, but selenium accumulations were about 2x greater in Idaho beetles than YNP (Fig. 2.6). Because YNP beetles accumulated less selenium in the exoskeleton even though the amount of selenium was lower in YNP than Idaho beetles, at least for selenium genetic differences between populations seems most likely.

Table 2.3. Main effects of year, location (Idaho vs. YNP) by mixed model analysis of metal weight in exoskeleton for As, Cd, Cu, Pb, Se (only significant effects are shown).

Element	Main effect	Num DF	Den DF	F Value	Pr > F
As	YNP	1	6	22.11	0.0033

Table 2.4. Main effects of year, location (Idaho vs. YNP) by mixed model analysis of metal concentration in exoskeleton for As, Cd, Cu, Pb, Se (only significant effects are shown).

Element	Main effect	Num DF	Den DF	F Value	Pr > F
As	YNP	1	2	10.04	0.0869

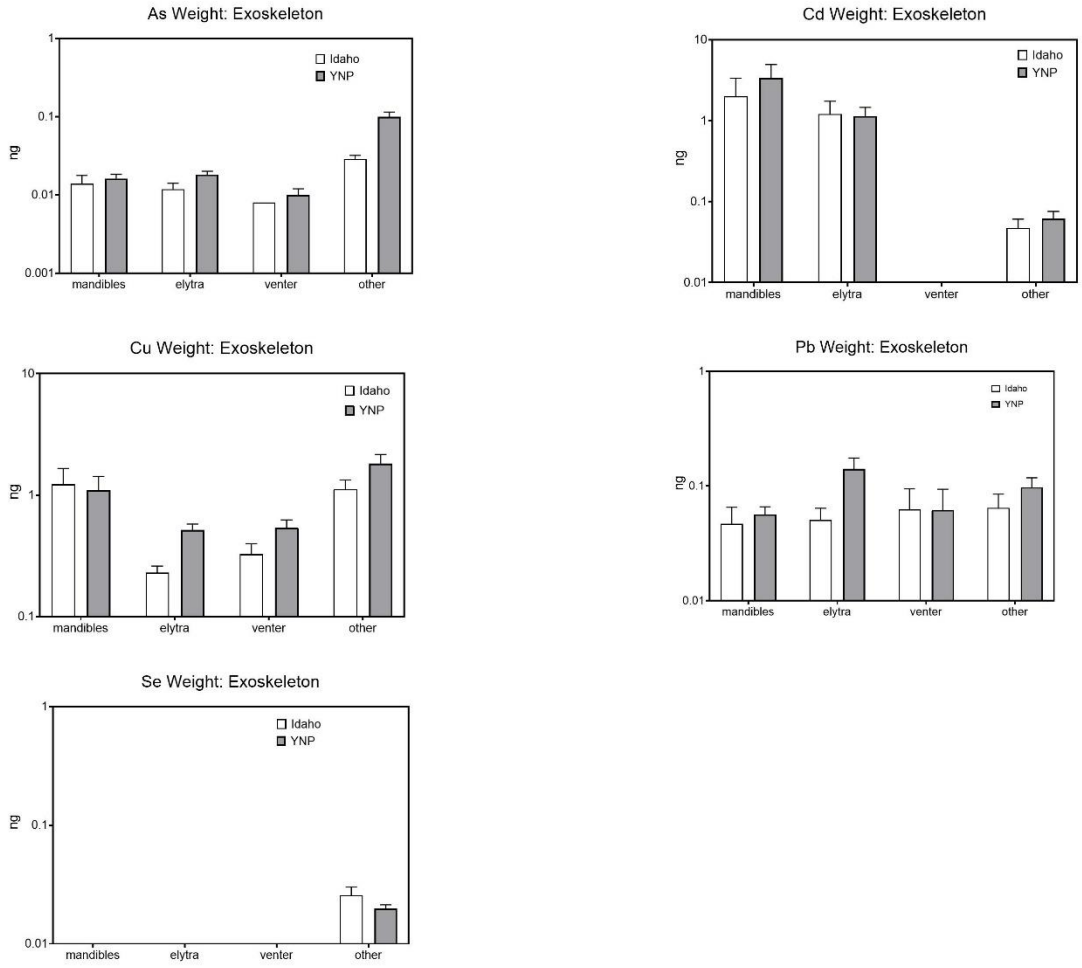

Figure 2.10. Distribution by weight (ng) of heavy metal contaminants in exoskeleton components (mandibles, elytra, venter, and others) of *Cicindelidia haemorrhagica* from Idaho (Loveridge site) and Yellowstone National Park (YNP, average of Dragon Hot Springs and Rabbit Creek) populations in 2018 – 2020. Means and standard errors based on n=4 per site, see methods for more details.

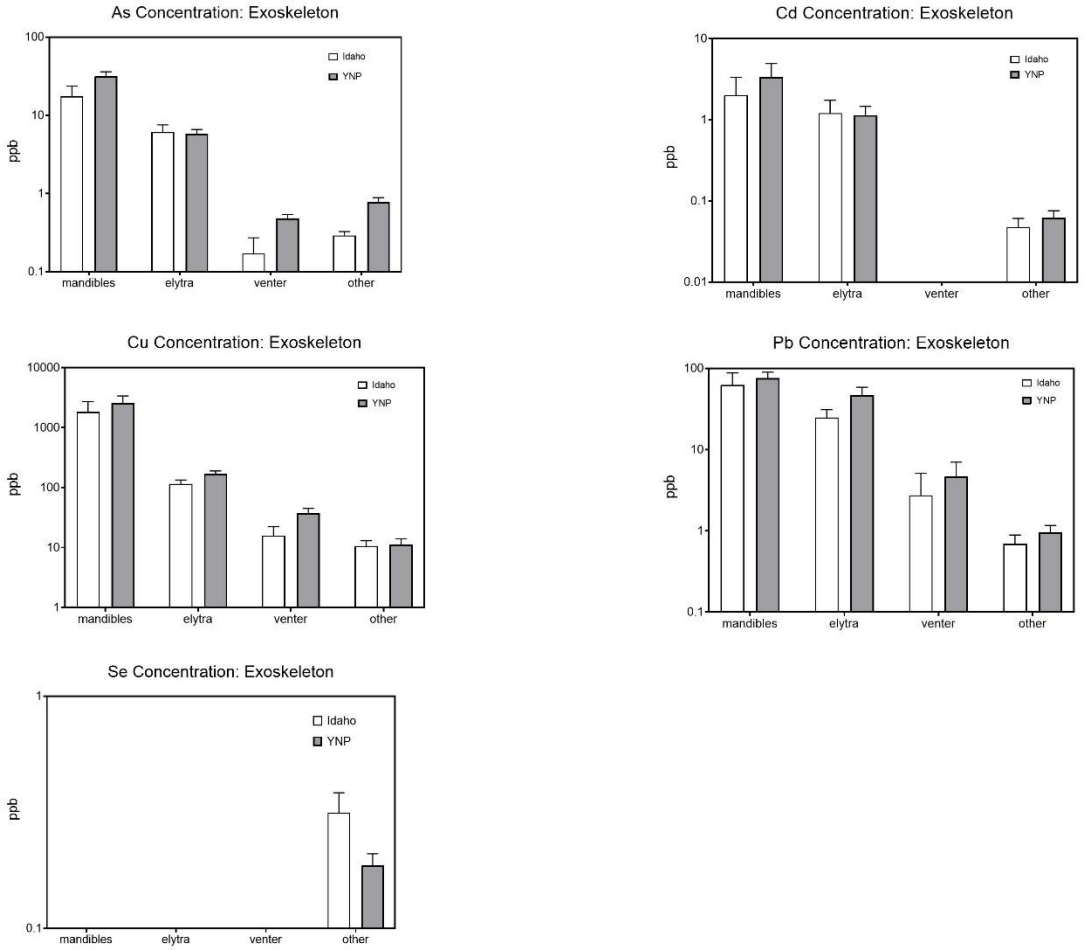

Figure 2.11. Total concentration (ppb = part (ng)/beetle wet weight (g)) differences in heavy metals among exoskeleton components (mandibles, elytra, venter, and others) of *Cicindelidia haemorrhagica* from Idaho (Loveridge site) and Yellowstone National Park (YNP, average of Dragon Hot Springs and Rabbit Creek) populations in 2018 – 2020. Means and standard errors based on n=4 per site, see methods for more details.

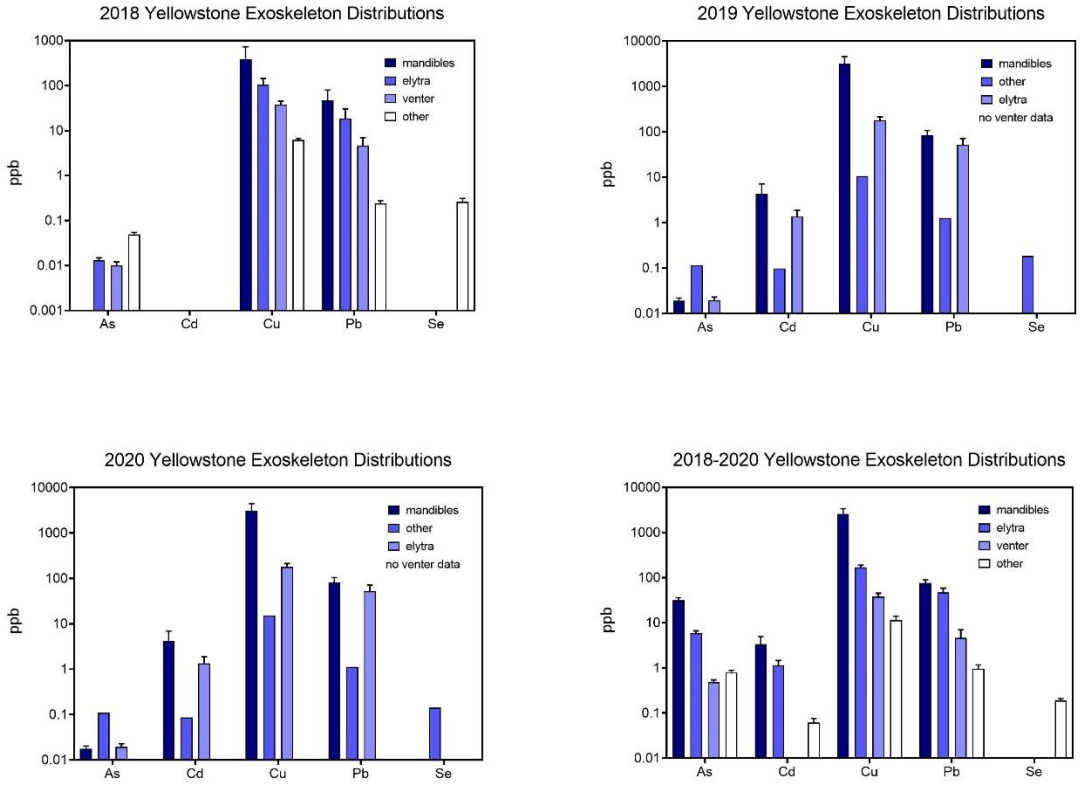

Figure 2.12. Concentration differences in heavy metals among exoskeleton components (mandibles, elytra, venter, and others) of *Cicindelidia haemorrhagica* from Yellowstone National Park (YNP, average of Dragon Hot Springs and Rabbit Creek) populations by year and averaged for 2018 – 2020. Means and standard errors based on n=4 per site, see methods for more details.

The distribution of metals and their concentrations among parts of the exoskeleton are show in Fig. 2.10 and Fig 2.11. Both Idaho and YNP populations show similar patterns of accumulation by weight and associated metal concentrations. Arsenic concentrations were greater in mandibles of YNP beetles in 2019 and 2020. Annual variation in concentration of metals by exoskeletal part is show for YNP beetles in Fig 2.12.

Discussion

I reviewed all sampling data for all years, and the differences were associated with ICP-MS values and not any other source (data entry or statistical error). Because the ICP-MS instrument was changed in 2020, and the 2019 samples were among the first samples processed with this instrument, and because we have no data on control samples for any metals, the 2019 values seem suspect. This problematic conclusion leaves open the question of which set of measurements are accurate: 2019 or 2018 and 2020?

Measurements within each year show acceptable precision (as illustrated by measured standard errors), so within-year comparisons are valid. However, the question of the accuracy of metal weights and concentrations cannot be resolved with our data to date. Previous research indicated that major heavy metal contaminants in YNP include antimony, arsenic, iron, and mercury (Qin et at. 2009). Additionally, as mercury occurred in its methylated form (Boyd et al. 2009), which is most biologically available, it seems other metals also might occur in biologically accessible forms. In my work, analytical tools to determine metal “species” (i.e., oxidation state and methylation) were not available. Also, the range of metals that could be determined was limited by available detectors on the ICP-MS at MSU (with which we had a pre-existing cooperative

agreement). Based on the combination of available detectors and biologically significant elements, I focused on arsenic (given its high toxicity and previous reports from YNP), cadmium (based on potential toxicity and available literature based on mine tailings), copper (based on its potential toxicity to insects at sufficiently high concentrations), lead (based on its importance as a vertebrate toxin and association with mining), and selenium (based on its toxicity and well-documented patterns of bioaccumulation in food chains).

I observed all five heavy metals in *C. haemorrhagica* from Idaho and YNP with arsenic and selenium showing the greatest differences between the two populations (Fig. 2.6 and 2.7., Table 2.1). Arsenic occurred in beetles at approximately three times the level in YNP beetles as compared to those in Idaho, and selenium occurred approximately three times higher in Idaho beetles as compared to those in YNP. These differences were reflected in the total concentrations per beetle, with arsenic concentrations in YNP beetles double those of Idaho beetles, and selenium concentrations in Idaho beetles almost double those of YNP individuals. Because YNP beetles accumulated less selenium in the exoskeleton even though the amount of selenium was lower in YNP than Idaho beetles, at least for selenium, genetic differences between populations seem most likely. However, RNA-sequencing and metabolomics data would be needed to resolve this question.

Higher concentrations of heavy metals where higher levels of those metals exist in the environment is expected. Dallinger & Rainbow (1993) emphasize that terrestrial invertebrates have evolved different methods for accumulating and eliminating metals, and that variation can occur among species and subspecies. Examining differences in bioaccumulations patterns between YNP and Idaho populations of *C. haemorrhagica*

illustrates whether such differences also occur in populations of a species or might offer evidence that the YNP populations represent a subspecies. Also, because Yellowstone was ice-covered during the Pinedale Glaciation (2.6 million years ago to 11,700 years ago, with ice sheets as deep as 1,600 m), the earliest *C. haemorrhagica* could have occupied what is now YNP is 11,700 years ago (Pierce 1979). Thus, any evolutionary divergence in YNP *C. haemorrhagica* based on exposure to elevated levels of heavy metals would have occurred over no more than 11,700 generations (assuming an annual life cycle).

Divergence in where specific metals are accumulated is indicated in Fig. 2.8 and Table 2.4. Broadly, YNP beetles seem to partition less metal in the exoskeleton than the Idaho beetles. Although arsenic was the only metal that showed statistically different accumulation in the exoskeleton. Other metals, particularly selenium, also indicate potential differences (Fig. 2.8) but given the relatively low number of beetles sampled, it seems likely random variation obscured these relationships. Because arsenic occurs in higher concentrations in YNP compared to Idaho, unlike other metals tested, this divergence in accumulation pattern is consistent with the argument of Dallinger & Rainbow (1993) that evolutionary changes in metal accumulation occur in high metal environments. This result also leads to the question of whether the YNP population may have diverged sufficiently to represent a new subspecies or species. Because Willemssens (2019) reports differences in thermoregulatory behaviors and other adaptations, my metal partitioning data add to evidence that *C. haemorrhagica* evolved in multiple ways to adapt to the unique environment of thermal areas in YNP.

When looking closer at where beetles accumulate heavy metals in parts of the exoskeleton, I examined the mandibles, elytra, abdominal venter, and remaining exoskeleton. Regarding the mandible, the mouthparts of many arthropods are known to be locations for greater concentrations of heavy metals which are cross-linked in an "organic matrix" (Sigel et al. 2008). Because tiger beetle elytra have structural coloration and also provide armored protection for the wings, this seems to be another logical location to examine differences in metal accumulation. Finally, the abdominal venter of *C. haemorrhagica* has an unusual (among tiger beetles) red-orange color, and on-going research (J. L. Bowley in Higley and Peterson laboratories) indicates that the venter may reflect infrared radiation (Bowley, J. L. unpublished), so differences in heavy metals accumulated in the venter might have a role in color or thermal reflectivity.

Metals were not uniformly distributed in exoskeletal tissue in either Idaho or YNP beetles (Fig. 2.10 – 2.12). The distribution of metals by weight and by concentration were largely similar between Idaho and YNP populations (Fig. 2.10), although selenium concentrations were higher in Idaho beetles. Regarding the concentrations of metals in different parts of the exoskeleton, the highest concentration of metals occurred in the mandibles, followed by the elytra, then the venter, and finally other exoskeletal tissues.

For example, in Fig. 2.11 and 2.12 the highest concentrations of copper are in the mandible. Copper is a component of several essential enzymes in plants and animals, but excess copper can be toxic because it alters copper redox reactions to produce superoxides and hydroxyl radicals (Tchounwou et al. 2012). According to Sigel et al. (2008), copper is used for biomineralization and heavy metal cross-linking which increases the local density. Tiger beetle mandibles are used for defense, prey capture,

holding prey, and by males to hold females during mating and mate guarding. These roles require strength and mandibles are among the most heavily sclerotized (hardened) parts of a tiger beetle as was obvious during my beetle dissections. Similarly, because the elytra and venter have important roles to protect vulnerable tissues, it is not surprising that metals might be used in those parts in a similar manner as in mandibles. What is not clear is whether metals have non-structural roles in any of these tissues.

Because metals occur in different, non-random concentrations in the exoskeleton, a mechanism or mechanisms must exist for moving metals into these parts. Also, based on previous research the precise methods for moving metals will differ depending on the metal or metal species (metal species are "toxic metals, metalloid compounds, and metal-based drugs …with endogenous ligands" Gailer 2013). Most commonly, heavy metals are transported bound to a metal-binding protein, which is specific to a given metal species (Hopkin 1989). However, more research into transport mechanisms is needed to determine whether *C. haemorrhagica* use metal-binding proteins, such as metallothioneins, to bind and store metals into cells for detoxification, or moved into membrane-bound granules, or if they are just being transported passively, through concentration gradients, or by diffusion (Dallinger & Rainbow 1993; Hopkin 1989).

The results with mandibles are consistent with the notion that some insects accumulate metals in the mandibles to provide increased structural strength. However, these results do not exclude the possibility that higher concentrations may be associated with sequestration of otherwise toxic levels of a metal in the exoskeleton, and these two hypotheses are not mutually exclusive. Hopkin & Martin (1984) and Clausen (1984) hypothesized that movement of metals into the exoskeleton could be a mechanism for

elimination during molting. However, when examining eight different species of arachnids (Clausen 1984) and *Lithobius variegatus* (centipede)C (Hopkin & Martin 1984), exuviae did not contain the zinc concentrations predicted, but instead concluded that zinc was associated with other tissues bound to the cuticle. Because my examination of *C. haemorrhagica* was of adults, metals in the exoskeleton would not be eliminated through molting because adult insects (except mayflies) do not molt.

Interestingly, because Clausen (1984) and Hopkin & Martin (1984) did not find zinc in molted cuticle, the non-living layer of the exoskeleton, it follows that exoskeletal zinc must be associated with integument, the living portion of the exoskeleton. Unless metals in the integument are granulated (the least energy expensive methods of sequestration for invertebrates) or isolated in cellular vacuoles or vesicles (Dallinger & Rainbow 1993; Hopkin 1989), high concentrations would still have the capacity to be toxic. My dissections did not separate cuticle from integument, but if Clausen's (1984) and Hopkin & Martin's (1984) findings are valid for insects and other heavy metals, then it seems less likely that movement of metals into the exoskeleton is a method of sequestration. Moreover, Fig. 2.11 and 2.12 show that metal concentration depends more on the part of the exoskeleton than on the total concentration of metal in the insect. For instance, although total arsenic concentrations were anywhere from four times (2018) to two times (2019, 2020) higher in YNP beetles than in Idaho beetles (Fig. 2.5, Table 2.1), the concentrations in mandibles, elytra, and venter were similar between YNP and Idaho. My findings are consistent with the interpretations of Clausen (1984) and of Hopkin & Martin (1984), that the exoskeleton is not being used by *C. haemorrhagica* for sequestration or elimination of heavy metals. Because past findings and my results

involve species in multiple arthropod classes (specifically, Chilopoda, Arachnida, and now Hexapoda), this phenomenon may be the general condition of terrestrial arthropods.

Is strengthening the exoskeleton the primary, or only mechanism for metal movement into the exoskeleton? And as this movement is against a concentration gradient, how is that movement regulated? I think it is unlikely that this question can be solved through field observations. However, if a heavy metal dose response study were conducted with a tiger beetle species in culture, it would be possible to more precisely determine the relationship between heavy metal concentration and exoskeleton partitioning and assays of metal-binding proteins and gene expression could provide insight into the regulation of this movement. Unfortunately, a well-recognized problem with laboratory assays of this sort, is that dosing, typically through a single species of easily reared prey, may not represent an accurate model of how beetles are intoxicated in natural environments. Consequently, there is a crucial need to understand metal movement from the environment, through prey species, and into *C. haemorrhagica* to determine important environmental and dietary factors associated with *C. haemorrhagica* response to heavy metals within the environment.

Beyond scientific issues, work with *C. haemorrhagica* and heavy metals has potential practical benefits. If mechanisms for *C. haemorrhagica* to bind metals are found, they could offer possible applications across many disciplines. In medicine, new metal-binding proteins might help save victims of heavy metal poisoning. In agriculture, new metal-binding agents might help restore contaminated soils and allow the safe use of these soils in crop production. Similarly, binding agents to remove metals in soil would help avoid ground water contamination. In sanitation, heavy metals can be important

contaminants of human and animal waste, so methods for removing these contaminants are essential. And as a form of bioremediation, new approaches for removing high concentrations of metals in mine tailings and industrial waste sites, as well as associated water sheds, continue to be needed.

Conclusion

As hypothesized, adult *C. haemorrhagica* in Yellowstone National Park bioaccumulate heavy metals, but so do *C. haemorrhagica* in Idaho. Cuticular waxes that showed concentrations of metals indicate that the adults are excreting the metals. The exoskeleton and internal tissues had substantially higher concentrations. Metal concentrations in the exoskeleton differed among metals but most statistically significant for arsenic and selenium. This significant difference in arsenic distribution in YNP beetles compared to Idaho beetles raises the question of whether *C. haemorrhagica* in YNP have so diverged in adaptations to metals and temperature associated with thermal areas that they may represent a distinct species or subspecies.

My work also shows the need for baseline data on insect toxicity to heavy metals, and especially to those of particular environmental concern including antimony, arsenic, cadmium, chromium, lead, and mercury. In addition, the roles of heavy metals in strengthening the exoskeleton, in the formation of exoskeletal structures, and in metal movement are largely speculative. My results show that patterns of total metal accumulation in the exoskeleton differ in YNP *C. haemorrhagica* compared to Idaho *C. haemorrhagica*, but whether or why these differences evolved is unknown. However, research in YNP presents considerable challenges. Researchers must cope with intense solar radiation plus heat from the thermal pools and vents, the mountainous terrain, the

thin crusts of heated soils in thermal areas, the high concentrations of human toxins in soil, water, and sometimes air, as well as challenges associated with meeting park regulations, wildlife, and unreasonable even reckless tourists. However, more researchers need to meet these challenges given the extraordinary opportunities to explore ecological and evolutionary questions, particularly with insects, in Yellowstone National Park.

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